Synaptic-Adhesion Molecules Neurexin 1 and Neuroligin 1 as Novel Prognostic Factors in Oral Squamous Cell Carcinoma

Hiromi Hirohata[1,2*]

Toru Yanagawa[3*]

Shohei Takaoka[1*]

Fumihiko Uchida[3]

Yoichiro Shibuya[4]

Satoru Miyabe[5]

Katsuhiko Tabuchi[6]

Yuichi Akagi[3]

Shogo Hasegawa[3]

Satoshi Sakai[7]

Yasutoshi Takeuchi[8]

Naomi Ishibashi-Kanno[3]

Kenji Yamagata[3]

Hiroki Bukawa[3]

[1]Department of Oral and Maxillofacial Surgery, Clinical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Ibaraki, Japan.

[2]Department of Oral and Maxillofacial Surgery, Tsukuba Central Hospital, Ushiku, Ibaraki, Japan

[3]Department of Oral and Maxillofacial Surgery, Faculty of Medicine, University of Tsukuba, Ibaraki, Japan

[4]Department of Plastic and Reconstruction Surgery, Faculty of Medicine, University of Tsukuba, Tsukuba, Ibaraki, Japan

[5]Department of Maxillofacial Surgery, Aichi-Gakuin University School of Dentistry, Nagoya, Japan

[6]Department of Neurophysiology, Faculty of Medicine, Shinshu University, Matsumoto, Nagano, Japan

[7]Department of Cardiovascular Medicine, Faculty of Medicine, University of Tsukuba, Tsukuba, Ibaraki, Japan

[8]Department of Oral and Maxillofacial Surgery, Mito Saiseikai General Hospital, Mito Ibaraki, Japan

Abstract

Background: Synaptic adhesion molecules regulate synapse development and maintenance. Neurexins and neuroligins have been implicated in psychiatric disorders, and shown that they are related to vascular system and carcinomas in recent studies. In the present study we focused neurexin 1 and neuroligin1, and investigated the relationship between those two molecules expression and clinical features of oral cancer.

Materials and methods: Fifty-six biopsy samples from oral squamous cell carcinomas (OSCCs) were analyzed semi quantitatively by immunohistochemistry. Correlations between the expression level of neurexin 1, neuroligin 1, p53, Ki67 and the clinical features of OSCCs were statistically analyzed.

Results: The neurexin 1 expression group contained significantly advanced T ($P < 0.05$) and N classification positive cases ($P < 0.01$) by univariate analysis. Overall survival was compared with Kaplan-Meier analysis and the log rank statistic. The prognosis of neuroligin 1 expression group was significantly more favorable ($P < 0.05$). A Cox proportional hazards model was used to test the significance of survival time, and N classification positive ($P < 0.05$), neurexin 1 ($P < 0.05$) and neuroligin 1 ($P < 0.01$) were predictive factor for survival.

Conclusions: Synaptic adhesive molecule could be useful prognosis factor for OSCC and may help to determine therapeutic policy.

Keywords

Neurexin 1; Neuroligin1; Oral squamous cell carcinoma; Head and neck

List of abbreviations

NRXN	:	Neurexin
NLGN	:	Neuroligin
OSCC	:	Oral Squamous Cell Carcinoma
RT	:	Radiation therapy
CT	:	Chemotherapy
SD	:	Standard Deviation

Introduction

The formation, activation, and degeneration of synapses are regulated by the orchestrated activity of thousands of molecules that localize to pre- and postsynaptic terminals. Synaptic-adhesion molecules are a diverse family of cell-adhesion molecules involved in the development and maintenance of synapses [1-2]. Synaptic-adhesion molecules called neurexins (NRXNs) and neuroligins (NLGNs) have been causally implicated in psychiatric disorders such as autism, schizophrenia, and drug addiction [3]. These proteins mediate signaling across the synapse, influence the properties of neural networks by synapse specificity, and play key roles in the pathogenic processes of various neurological disorders [4].

Recent studies show that synaptic cell-adhesion molecules are also involved in the vascular system. NRXN and NLGN are widely expressed in the vascular system and are dynamically regulated in the vascular system [5]. Neuroligin and β-neurexin synergize with extracellular-matrix-binding vascular endothelial-growth factor A (VEGF-A) during vascular development [6]. Although neuroligin 1 (NLGN1) does not affect endothelial-cell proliferation or migration, it cooperates with α6 integrin to modulate the vessel-stabilizing protein laminin [7]. Deleting NLGN1 causes an aberrant distribution of VE-cadherin, laminin, and α6 integrin in blood vessels, along with significant structural defects in the vascular tree [7]. Since tumor blood vessels promote tumor progression by supplying nutrition and oxygen to the tumor and acting as gatekeepers, the synaptic adhesion molecules involved in the vasculature might also affect the status of oral cancer [8].

Neuroligin3 (NLGN3) has been identified as a leading candidate mitogen, and soluble NLGN3 is both sufficient and necessary to promote cell proliferation in high-grade gliomas [9]. NLGN3 induces PI3K-mTOR pathway activity and feed-forward NLGN3 expression in glioma cells, and the NLGN3 expression level in human high-grade glioma is negatively correlated with overall patient survival [9]. Fox Q1 directly binds the NRXN3 promoter region to suppress its activity in gliomas, and promotes glioma-cell proliferation and migration by down regulating NRXN3 [10]. NRXN3 gene mutation is significantly associated with an elevated risk of developing breast cancer [11].

Although these reports suggest a close relationship between synaptic-adhesion molecules and cancers, most NRXN and NLGN research has focused on neuroscience. Thus, the expression of these molecules in oral cancer has not been characterized. To determine whether NRXNs and NLGNs are suitable prognostic indicators for oral cancer, we here examined NRXN1 and NLGN1 expression in OSCCs and the relationship between these molecules and OSCC clinico-pathological features.

Methods

Patient Characteristics

Tumor specimens were obtained by surgical biopsies of tongue carcinoma from 56 patients who consulted the Department of Oral and Maxillofacial Surgery at the University of Tsukuba Hospital between 2007 and 2012. The study included 27 males and 29 females with a median age of 70; the range was 23–87 years. Each tumor specimen was staged according to the International Union against Cancer (UICC) system, and the composition of the cases is shown in Table 1 [12]. Biopsies were obtained prior to any treatment for the malignancy. The patients were treated by surgical excision alone (40

patients), surgical excision with chemo radiotherapy (10 patients), chemo radiotherapy alone (2 patients), or radiotherapy alone (4 patients). The 16 patients treated with radiotherapy were treated by LINAC (26–100Gy). Informed consent was obtained prior to obtaining specimens, and the study protocol was reviewed and approved by the Research Ethics Committee of the University of Tsukuba (H25-43).

Immunohistochemistry

Tissue samples were immune stained using the En Vision FLEX system (Dako: Agilent Technologies Japan, Ltd. Tokyo, Japan) according to the instruction manual, as described previously [13]. Specimens were fixed with 10% neutral buffered formalin, embedded in paraffin, and sectioned at 5 μm. The antigens were activated with citric acid and autoclaved. The NRXN1 rabbit polyclonal antibody was originally generated and provided by M.W., and was diluted 1:1000 v/v in PBS for use. The NLGN1 antibody (Affinity Bioreagents, Inc., Golden, USA) was diluted 1:50 v/v in PBS. The p53 and Ki67 rabbit monoclonal antibodies (Cell Signaling Technology, Inc., Danvers, USA) were diluted 1:160 and 1:400 v/v in PBS; tissue sections were incubated in the antibody solution at room temperature for 1 hour. Human brain sections were used as positive controls for NRXN1 and NLGN1. Sections were reacted with biotinylated goat anti- rabbit IgG antibody and then with horse radish peroxidase-conjugated streptavidin, and were visualized with 3,3'-diaminobenzidine. The immune stained slides were examined by a pathologist (S.M.) who was not informed of the patient's clinical status. Tissues were categorized as positive or negative for NRXN1, NLGN1, and p53. Ki67 immunostaining was evaluated using the MIB1 index.

Statistical Analysis

For univariate analysis, we used Student's t-test, Fisher's exact probability test, and the chi-square test. Kaplan-Meier analysis was tested by log-rank. For multivariate analysis, we used a Cox proportional hazard regression model. Statistical analyses were performed using the software package JMP 12.0.1 for Mac (SAS Institute Inc. Cary, NC, USA). Backward-variable selection was performed to examine which factors remained independent indicators of survival.

Results

Univariate analysis of correlations between NRXN1 and NLGN1 expression and clinico-pathological features

Based on immune staining, tumors were classified as NRXN1-positive (6 of 56; 10.7%) or NRXN1-negative (50 of 56; 89.3%), and asNLGN1-positive (17 of 56; 30.4%) or NLGN1-negative (39 of 56; 89.3%) (Figure 1 and 2). The tumors were further classified according

Neurexin 1
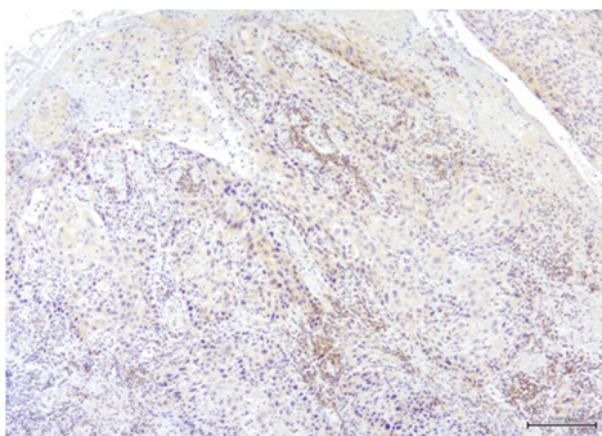
x40
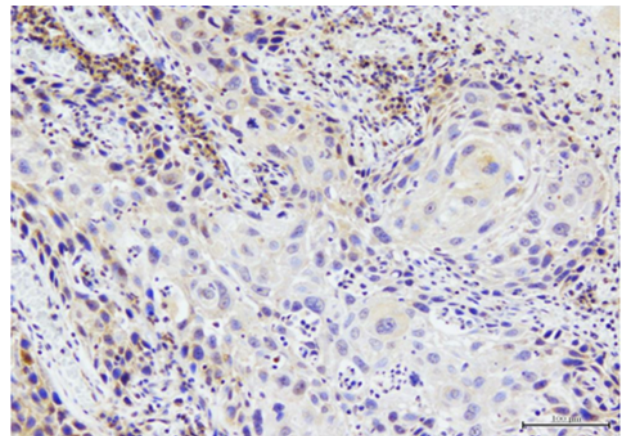
x100

Figure 1: Representative photomicrographs of immunohistochemical staining with a neurexin 1 antibody show diffuse and intense nuclear and cytoplasm staining in tumor tissues and parts of lymphocytes. Original magnification: upper panel40×; lower panel 100×.

Neuroligin1

x40

x100

Figure 2: Representative photomicrographs of immunohistochemical staining with a neuroligin 1 antibody. Cytoplasmic staining is more intense in tumor nests. Original magnification: upper panel 40×; lower panel 100×
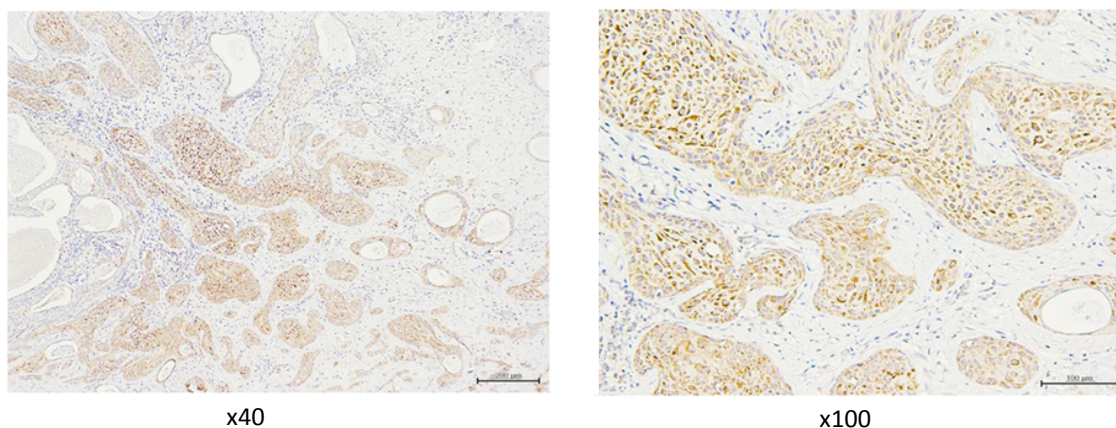
		n
Median age		70
Range		23-87
Sex ratio f/m		27/ 29
TNM classification		
T category	T1	14
	T2	23
	T3	9
	T4a	10
N category	N0	40
	N1	6
	N2a	0
	N2b	8
	N2c	1
	N3	1
M category	M0	56
	M1	0
Stage	I	13
	II	18
	III	9
	IV	16
Differentiation		
	G1	37
	G2	6
	G3	13
	G4	0
Lesion		
	Tongue	31
	Gingiva	14
	Buccal mucosa	6
	Lip	2
	Maxillary sinus	1
	Palate	1
	Floor of mouth	1
Therapy	Surgery alone	40
	RT + CT + Surger	10
	RT + CT	2
	RT	4
Total		56

Table 1: Characteristics of oral squamous cell carcinoma patients

to TNM categories: for T, tumors were classified as either T1/T2 or T3/T4, and for N and p N, tumors were classified as negative (N0) or positive (N1–3) for lymph-node metastasis. The tumors were also divided by clinical stage (I/II or III/IV) and as moderately or poorly differentiated (G2/G3) or well-differentiated (G1).

We found significant correlations between NRXN1 expression and various clinico-pathological characteristics (Table 2). The NRXN1-positive group included significantly more males (P < 0.05) and alcohol drinkers (P < 0.05), and more cases with an advanced T category (P < 0.01) or N category (P < 0.01) or treatment by radiotherapy (P < 0.05). There was no significant difference in NRXN1expression with respect to any other factor, including age, clinical stage, and location of the lesion, mode of invasion, MIB1 index, p53 expression, or NLGN1 expression.

There were no significant correlations betweenNLGN1 expression and any other factor, including age, sex, alcohol consumption, smoking, T or N category, clinical stage, differentiation, location of the lesion, type of therapy, mode of invasion, MIB1 index, p53 expression, or NRXN1 expression.

Overall survival relative to the expression of synapse-adhesion molecules we used Kaplan-Meier curves to show overall survival relative to the expression of the synaptic adhesion molecules NRXN1 or NLGN1 (Figure 3). Log-rank analysis did not show any significant difference in the 5-year survival rate in theNRXN1-positive (1.00) and NRXN1-negative (0.755) groups (P = 0.27). However, there was a significant difference in 5-year survival rates for theNLGN1-positive (1.00) and NLGN1-negative (0.703) groups (P < 0.05).

Multivariate Analysis of Overall Survival in the Absence or Presence of Synapse-Adhesion Molecules

We used a Cox proportional hazards regression model to analyze correlations between NRXN1 and NLGN1 expression and overall survival rate; Table 3 shows the hazard ratios (HR) and 95% confidence intervals (CI).

We adopted all of the variables except for the clinical stage, which was removed because of a remarkable number of confounding factors, and investigated significant covariates by fitting to the Cox proportional hazards model. Backward-variable selection was performed and 8 variables as followings were selected. NLGN1 expression was forced into variables. Cases except age were divided into two groups according to each of the following: smoking (yes or no), alcohol consumption (yes or no), N category (N1–3: N0), Ki67 index ($\leq 110 \leq$: >110), p53 expression (positive or negative), NRXN1 expression (positive or negative), and NLGN1 expression (positive or negative).

Lymph-node metastasis, the absence of NRXN1 expression, and the absence of NLGN1 expression were significantly associated with an33.24–fold increase in a positive N classification (HR = 33.24; 95% CI = 4.29–759.05, P > 0.001), a 0.07–fold decrease in the presence of NRXN1 expression (HR = 0.07; 95% CI = 0.0039–0.986 P < 0.05), and a 1.25 x10-10–fold decrease in the presence of NLGN1 expression (HR = 1.25 x10-10; 95% CI = 0–0.31, P < 0.01). No other variables were significantly associated with overall survival.

Discussion

In the present study, we found that associations between the expression of NRXN1 and NLGN1andvarious clinical features in OSCC. Univariate analysis showed that the NRXN1-positivegroup included significantly more males (P < 0.05) and people who drink alcohol (P < 0.05), more cases with an advanced T category (P < 0.01) or N category (P < 0.01), and more cases that were treated by radiotherapy (P < 0.05). However, there was no significant difference between the NLGN1-positive and NLGN1-negative groups. Although we found a correlation between NRXN1 and radiotherapy, the NRXN1 could not have been induced by radiation, because the specimens were obtained prior to treatment. Rather, this finding reflects the fact that the NRXN1-positive group included more tumors with an advanced T or N category, which are more likely to be treated by radiotherapy. Kaplan-Meier and log-rank analyses did not show any significant differences between the NRXN1-positive and NRXN1-negative groups (P = 0.27), but showed a significantly more favorable prognosis for the NLGN1-positive group than the NLGN1-negative group (P < 0.05). Notably, the 5-year survival rate was 1.0 for both the NRXN1-positive and NLGN1-positive groups. In considering these results, it is important to note that NRXN1 was present in only 10.7% of the tissue samples (6 of 56), and it will be necessary to study a larger number of NRXN1-positive samples for more precise analysis. Cox regression analysis of overall survival, indicated that a positive N classification (P > 0.001), NRXN1 expression (P = 0.05), and NLGN1 expression (P < 0.01) were predictive factors for overall survival (Table 3). And there was tendency to associated with alcohol consumption (P=0.08). Our finding that the N classification predicts poor survival is reasonable, and is consistent with previous reports of the clinical significance of cervical lymph-node metastasis [14-16]. Our results also agree with many reports on the association between alcohol consumption and mortality and carcinogenesis, and with other reports indicating that alcohol is related to a poor prognosis [17-22]. In the absence of lymph-node metastasis, NRXN1 and NLGN1 can be predictive factors for overall survival. On the other hand, neither the NRXN1 nor NLGN1 expression was significantly related to ki67 or p53 expression in our results (Tables 2 and 4). Thus, NRXN1 and NLGN1 cannot be related
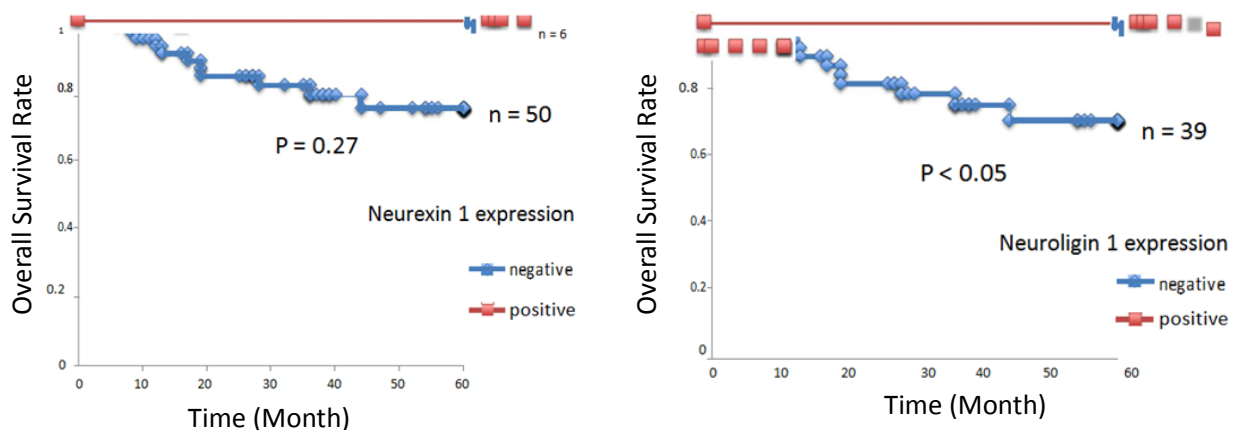

Figure 3: Kaplan-Meier estimates of the overall survival in oral squamous cell carcinoma cases categorized by the expression of the synapse-adhesion molecules neurexin 1 (upper graph) and neuroligin 1 (lower graph).

	Negative	Positive	P value	
Age				
	66.14	60.33	0.35	t
Sex				
f	27	0	< 0.05 f	
m	23	6		
Alcohol				
no	32	1	< 0.05 f	
yes	17	5		
smoking				
no	29	3	0.69	f
yes	20	3		
T category				
T1+2	36	1	< 0.05 f	
T3+4	14	5		
N category				
N0	39	1	< 0.01 f	
N1+2	11	5		
Stage				
I+II	30	1	0.08	f
III+IV	20	5		
Differentiation				
G1	35	2	0.17	f
G2+3	15	4		
Lesion				
Tongue	28	3	0.65	c
Gingiva	13	1		
The others	9	2		
Therapy				
Surgery alone	38	2		
with RT	12	4	< 0.05 f	
Mode of invasion				
01-Mar	29	3	1	f
4	21	3		
Ki67 index				
	169.4	154.2	0.84	t
p53 expression				
negative	30	3	0.68	f
positive	20	3		
Neuroligin expression				
negative	33	6		
positive	17	0	0.16	f

Table 2: Correlation with neurexin and clinical feature

	Hazard Ratio	95% CI	P value
Age (by 1 year)	1.05	0.99 - 1.12	0.12
Smoking (Yes : No)	2.58	0.51 - 14.51	0.26
Alcohol (Yes : No)	6.63	0.79 -86.48	0.08
N category (N1-3:N0)	33.24	4.29 - 759.05	< 0.001
Ki67 index (110 ≤ : 110>)	1.63	0.430- 7.84	0.48
p53 expression (Positive: negative)	0.3	0.042-1.35	0.12
Neurexin expression (Positive: Negative)	0.07	0.0039- 0.986	< 0.05

Table 3: Cox regression analysis of overall survival

	Negative	Positive	P value	
Age				
	67.85	60.18	0.07	t
Sex				
f	20	7	0.57	f
m	19	10		
Alcohol				
no	22	11	0.77	f
yes	16	6		
Smoking				
no	23	9	0.77	f
yes	15	8		
T category				
T1+2	25	12	0.76	f
T3+4	14	5		
N category				
N0	26	14	0.34	f
N1+2	13	3		
Stage				
I+II	20	11	0.4	f
III+IV	19	6		
Differentiation				
G1	24	13	0.36	f
G2+3	15	4		
Lesion				
Tongue	21	10	0.6	c
Gingiva	9	5		
The others	9	2		
Therapy				
Surgery alone	26	14		
with RT	13	3	0.33	f
Mode of invasion				
1-3	22	10	1	f
4	17	7		
Ki67 index				
	176	146.5	0.56	t
p53 expression				
negative	24	9	0.56	f
positive	15	8		
Neurexin expression				
negative	33	17		
positive	6	0	0.16	f

Table 4: Correlation with neuroligin expression and clinical feature

to cell proliferation or to a mutation of the tumor suppressor gene.

Although it is not certain how NRXN1 and NLGN1 expression are associated with prognosis, the association might be related to angiogenesis. The synaptic proteins NRXN and NLGN are widely expressed in the vascular system and are dynamically regulated in the vascular system [5]. A peptide from the extracellular region of the synaptic protein neurexin stimulates angiogenesis and the vascular-specific tyrosine kinase Tie2 [23]. Tie2 is expressed in the endothelium of neo vessels in regenerating organs and in several types of cancer, including leukemia, breast, gastric, thyroid, and oral cancers, and the expression of Tie2 in oral cancer is associated with lymph-node metastasis [24]. One report indicates that angiogenic markers in OSCC proteins are prognostic [25]. Although further study is necessary to clarify their association with angiogenesis, NRXN1 and NLGN1 might in some way regulate the vasculature of cancer nest cells.

A second possibility is that NRXN1 and NLGN1 are associated with prognosis through their involvement in cell adhesion. Synaptic-adhesion molecules belong to the family of cell-adhesion molecules, many of which are involved in OSCC [26]. The expression of cell-adhesion molecules is altered in OSCC, and the loss of their expression is often seen in poorly differentiated lesions [27]. Certain integrins are consistently up regulated in OSCC, suggesting that integrins may play an active role in disease progression [27]. Integrins are also synaptic-adhesion molecules and are clearly important in the invasive process, whereas intercellular adhesion receptors restrain invasion

and promote a more differentiated phenotype [2,28]. Furthermore, cell-adhesion molecules are used as molecular markers to predict tumor invasion, metastatic potential, and prognosis [29]. If NRXN1 and NLGN1play roles similar to those of cell-adhesion molecules in OSCC, they may alter tumor dynamics.

Given the relatively small number of samples in this study, the usability and mechanisms of NRXN1 and NLGN1 as prognostic factors in OSCC should be clarified with a larger number of samples. However, the use of the synaptic-adhesion molecules NRXN1 and NLGN1 is a novel method for evaluating clinical cancer status, and this is the first study to evaluate their prognostic value in oral (or any other) cancer. Our findings indicate that it may be possible to establish NRXN1 and NLGN1 as novel prognostic markers for oral cancer. If we can also clarify the mechanism, it may be possible to use these molecules as both tumor markers and novel therapeutic targets.

Conclusion

By analyzing 56 samples of OSCC patients we characterized the expression profiles of NRXN1 and NLGN1 in OSCC. Kaplan-Meier and log-rank analyses revealed a more favorable prognosis only for the NLGN1-positive group. Cox regression analysis indicated a favorable prognosis for abstinence from alcohol, the absence of lymph-node metastasis (N0), and the presence of NRXN1 and NLGN1. Thus, our results indicate that NRXN1 and NLGN1 are novel predictive factors for overall survival in OSCC. These markers should prove useful when selecting therapeutic strategies and their utility should be confirmed by further studies. It may also be possible to develop NRXN1 and NLGN1 as potential therapeutic targets by clarifying their activity and association with prognosis in OSCC.

Declarations

Ethics Approval and Consent to Participate:

Ethical approval for this study and for the use of existing samples was granted (No. H25-43: the Research Ethics Committee of the University of Tsukuba).

Availability of Data and Materials:

All the data that support the conclusions of this article is stored in the Tsukuba Paper Registration System.

Competing Interests:

The authors declare that they have no competing interests.

Consent for Publication:

Not applicable.

Author's Contributions

HH,TY, TS, NIK, YA, SS, and FU collected the data. TY, HH and TS drafted and wrote the manuscript. TY, KY, SH, YS, KA,YT, HH, and HB performed the surgeries. SM and KT performed histological analysis and interpreted the data. The concept of this manuscript was devised by TY. All authors read and approved the final manuscript.

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